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June 2, 1994

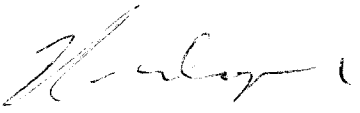
Philip Migliore, M.D.
Research Director
Moran Foundation

Dear Dr. Migliore:

Enclosed please find my progress report for Moran Foundation Project number 0066. I am very pleased with the progress that has been made on this work. Let me express my appreciation for the support provided by the Moran Foundation.

If you have any questions, please feel free to contact me.

Sincerely,



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Thomas A. Cooper, M.D. PROGRESS REPORT 6/2/94

MORAN FOUNDATION PROJECT 0066
Selection of Exon Sequences That Facilitate Pre-mRNA Splicing

The aims of this proposal are to: i) establish an in vivo system to select for exon sequences that enhance splicing and (ii) test individual sequences for splicing enhancing activity in two different vectors.

We have made significant progress toward the goals of this project. The scheme as outlined in the original proposal is to insert a DNA fragment containing 10-13 positions of random sequence into the middle exon of a minigene derived from human ß-globin, express this gene in tissue culture cells, amplify sequence from mRNAs that include the "random exon" to identify those sequences that enhance splicing. We hypothesize that different classes of sequences that enhance splicing of the middle exon will be found. Our specific approach is to molecularly clone a synthetic DNA fragment into the minigene by ligation then transiently transfect the total ligation into tissue culture cells and amplify the sequence of the spliced mRNA by reverse transcriptase-PCR (RTPCR). We are essentially testing millions of sequences for splicing-enhancing activity and each sequence is present in only several thousand copies. Our procedures therefore need to be extremely efficient and our assays highly sensitive. Therefore, our primary focus has been to develop high efficiency ligation and transfection procedures and sensitive RTPCR procedures.

First, we determined that a synthetic DNA fragment containing 13 random positions was of sufficient size to visualize and gel-isolate following restriction digests. Second, to make the DNA containing the 13 random positions double stranded, we found that PCR was a more efficient approach than a straight "fill in" approach using a single priming oligonucleotide as originally proposed. Third, our initial tests have determined that ligation efficiencies need to be improved 10 fold to yield sufficient levels of RNA following transient transfection to be detected by RTPCR. A summer student in the lab is currently working to optimize the following parameters to improve ligation: (i) preparation of plasmid vector DNA; (ii) preparation of insert DNA; (iii) ratio of plasmid to insert; (iv) effect of phosphatase treatment of the insert; (v) ligation conditions.

We have initiated a pilot "run through" of the scheme outlined in the original proposal to identify and address potential problems. The random exon was ligated into the plasmid vector and instead of transfecting directly into eukaryotic cells, the plasmid was transfected into bacteria and plated to form colonies. To confirm that a random population of inserts was cloned into the plasmid vector, plasmid DNA from 10 colonies was sequenced and all were found to be unique. Several hundred colonies were pooled, inoculated into 500 ml of bacterial broth, and used to generate a large prep of plasmid DNA. This plasmid preparation, which contains a pool of hundreds of exon sequences, was then transfected into eukaryotic cells and the RNA was assayed by primer extension. As expected, only mRNAs that skip the middle exon was detected by primer extension since those molecules that may enhance inclusion are present in very low numbers. This result confirms that the majority of random sequences are "neutral" with regard to exon inclusion. This is an important result indicating that background level of exon inclusion is low so that those sequences amplified from mRNAs containing the middle exon will most likely contain splicing enhancer sequences. We will next perform RTPCR on the RNA isolated from transfected cells to determine whether exon-including RNAs will be detected.

This project is still active and preliminary results were recently presented at the RNA Processing Meeting in Madison, WI, May 24-29, 1994. I plan to include the results from these preliminary studies in a proposal to obtain funding from an outside agency.